

RGS2 blocks slow muscarinic inhibition of N-type Ca²⁺ channels reconstituted in a human cell line

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1. Native N-type Ca²⁺ channels undergo sustained inhibition through a slowly activating pathway linked to M1 muscarinic acetylcholine receptors and G α q/11 proteins. Little is known concerning the regulation of this slow inhibitory pathway. We have reconstituted slow muscarinic inhibition of N-type channels in HEK293 cells (a human embryonic kidney cell line) by coexpressing cloned α 1B (Ca_v2.2) Ca²⁺ channel subunits and M1 receptors. Expressed Ca²⁺ currents were recorded using standard whole-cell, ruptured-patch techniques.
2. Rapid application of carbachol produced two kinetically distinct components of Ca²⁺ channel inhibition. The fast component of inhibition had a time constant of < 1 s, whereas the slow component had a time constant of 5–40 s. Neither component of inhibition was reduced by pertussis toxin (PTX) or staurosporine.
3. The fast component of inhibition was selectively blocked by the G $\beta\gamma$ -binding region of β -adrenergic receptor kinase 1, suggesting that fast inhibition is mediated by G $\beta\gamma$ released from G α q/11.
4. The slow component of inhibition was selectively blocked by regulator of G protein signalling 2 (RGS2), which preferentially interacts with G α q/11 proteins. RGS2 also attenuated channel inhibition produced by intracellular dialysis with non-hydrolysable GTP γ S. Together these results suggest that RGS2 selectively blocked slow inhibition by functioning as an effector antagonist, rather than as a GTPase-accelerating protein (GAP).
5. These experiments demonstrate that slow muscarinic inhibition of N-type Ca²⁺ channels can be reconstituted in non-neuronal cells, and that RGS2 can selectively block slow muscarinic inhibition while leaving fast muscarinic inhibition intact. These results identify RGS2 as a potential physiological regulator of the slow muscarinic pathway.

Muscarinic inhibition of N-type calcium (Ca²⁺) channels has been extensively studied in rodent superior cervical ganglion (SCG) neurons. Inhibition of N-type channels occurs through several different signalling pathways linked to one or more subtypes (M1–M5) of muscarinic receptor. The best understood pathway is fast and produces a voltage-dependent, membrane-delimited form of channel inhibition (Hille, 1994; Dolphin, 1998; Ikeda & Dunlap, 1999). This fast pathway involves signalling by G $\beta\gamma$ dimers (Herlitze *et al.* 1996; Ikeda, 1996) that are activated through M2 receptors in mouse (Shapiro *et al.* 1999) and through M4 receptors in rat (Bernheim *et al.* 1992; Fernandez-Fernandez *et al.* 1999). A second, less understood pathway generates fast, voltage-independent inhibition (Beech *et al.* 1992). Recent experiments indicate that this second fast pathway involves signalling by both G $\beta\gamma$ and G α (Kammermeier *et al.* 2000).

A third muscarinic pathway generates slow, voltage-independent inhibition of N-type Ca²⁺ channels (Bernheim *et al.* 1991; Beech *et al.* 1992). Slow muscarinic inhibition requires the activation of M1 receptors (Bernheim *et al.* 1992; Shapiro *et al.* 1999) and involves the production of an unknown, diffusible messenger (Hille, 1994). M-type potassium (K⁺) channels are also inhibited through a slow pathway linked to M1 receptors. However, it remains uncertain whether this inhibition occurs through the same slow pathway that inhibits N-type Ca²⁺ channels. Thus, recent experiments indicate that slow muscarinic inhibition of M-type K⁺ channels primarily depends on G α 11, whereas slow inhibition of N-type Ca²⁺ channels mainly involves G α q (Haley *et al.* 2000). Because slow inhibition is sustained, slowly reversible, and cannot be relieved by repetitive depolarization, it is likely to exert a profound influence upon neuronal excitability (Hille, 1994; Ikeda & Dunlap,

1999). It is therefore important to identify factors that can regulate the slow inhibitory pathway.

Regulator of G protein-signalling (RGS) proteins are a recently discovered group of molecules that interact with heterotrimeric G proteins. RGS proteins each contain a recognizable core domain that binds the switch regions of certain G α subunits (Tesmer *et al.* 1997; Berman & Gilman, 1998). RGS proteins are usually assumed to function as GTPase-accelerating proteins (GAPs) and to be responsible for rapid deactivation of G protein-dependent pathways (Chen *et al.* 2000; De Vries *et al.* 2000). By accelerating GTP hydrolysis, RGS proteins speed reformation of inactive G α G β heterotrimers and thereby attenuate signals transmitted by both G α and G β subunits (Berman & Gilman, 1998). However, RGS proteins can also function as 'effector antagonists' by binding to G α subunits and physically blocking their interactions with downstream signalling molecules (Hepler *et al.* 1997; Yan *et al.* 1997). As effector antagonists, RGS proteins block signalling by G α but not G β . The relative importance of effector antagonism *vs.* GAP activity has been little explored. Distinguishing between these two behaviours of RGS proteins requires a system in which signalling by both G α and G β can be monitored. N-type Ca²⁺ channels provide such a system because they are inhibited in distinct fashions by G α and G β subunits (Ikeda & Dunlap, 1999).

Recent studies have suggested that RGS proteins play important roles in Ca²⁺ channel physiology (Jeong & Ikeda, 1998, 2000; Diversé-Pierluissi *et al.* 1999; Melliti *et al.* 1999, 2000; Chen & Lambert, 2000; Mark *et al.* 2000). The goal of the present work was to determine whether an RGS protein can regulate slow muscarinic inhibition of N-type Ca²⁺ channels. Toward that end, we reconstituted slow muscarinic inhibition in HEK293 cells. In this system, the identity of the receptor, the channels, and the RGS protein could be controlled. We found that RGS2 selectively blocks slow muscarinic inhibition of N-type channels, while leaving fast inhibition intact or even enhanced. These results provide new insight into RGS protein function, and identify RGS2 as a potential physiological regulator of the slow muscarinic pathway.

METHODS

Cell culture and transfection

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and propagated in a medium of 90% Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum and 50 μ g ml⁻¹ gentamicin. The cells were trypsinized weekly and replated onto 60 mm culture dishes at ~20% confluence. CaPO₄ precipitation (CellPect kit, Pharmacia) was used to transfect these cells within 3–5 days. The transfection mixture contained expression plasmids encoding α 1B (Ca_v2.2), α 2- δ and β 3 Ca²⁺ channel subunits at 1.25 μ g each cDNA per dish, plus the M1 muscarinic acetylcholine receptor at 0.25 μ g cDNA per dish. In selected experiments, a plasmid encoding the carboxyl-terminus region (Gly⁴⁹⁵–Leu⁶⁸⁹) of β -adrenergic receptor kinase 1 (denoted β ARK1ct) was cotransfected

at 1.25 μ g cDNA per dish. In other experiments, a plasmid encoding RGS2 (fused in-frame to the carboxyl-terminus end of enhanced green fluorescent protein; EGFP) was cotransfected at 0.625 μ g cDNA per dish. For transfections that did not include the EGFP–RGS2 fusion construct, a separate plasmid encoding EGFP was included at 0.125 μ g cDNA per dish. The day following transfection, cells were briefly trypsinized and replated onto 12 mm round glass coverslips. Electrophysiological experiments were performed 16–24 h later. Successfully transfected cells were visually identified by their green fluorescence under ultraviolet illumination. Recordings were made exclusively from fluorescent cells.

Expression plasmids

cDNA encoding the rabbit α 1B (GenBank D14157) was in the expression vector pKCRH2. Rat α 2- δ (M86621) was in pMT2 (Genetics Institute, Cambridge, MA, USA). Rabbit β 3 (X64300) was in pcDNA3 (Invitrogen). Human M1 muscarinic acetylcholine receptor (X52068) was in pCD (Okayama & Berg, 1983). Jellyfish enhanced green fluorescent protein (U55763) was in pEGFP (Clontech). Human RGS2 (L13463) was in pEGFP-C2 (Clontech). β ARK1ct (M34019) was in pRK5 (Koch *et al.* 1994).

Patch-clamp recordings

Large-bore patch pipettes were pulled from 100 μ l borosilicate glass micropipettes (VWR 53432–921) and filled with an internal solution containing (mM): 155 CsCl, 4 Mg-ATP, 0.32 Li-GTP and 10 Hepes, pH 7.4 with CsOH. This pipette solution also contained either 0.1 Cs₂EGTA, 10 Cs₂EGTA or 20 Cs₄BAPTA as indicated. The final concentration of CsCl was adjusted to maintain constant osmolarity among the internal solutions. For experiments illustrated in Fig. 6, GTP was replaced by 0.32 mM GTP γ S. Aliquots of the pipette solutions were stored at –80°C, kept on ice after thawing and filtered at 0.22 μ m immediately before use. Internal solutions containing GTP γ S were used within 4 h of thawing. Pipette tips were coated with paraffin to reduce capacitance and then fire-polished. Filled patch pipettes had DC resistances of 1.0–1.5 M Ω . The bath solution contained (mM): 145 NaCl, 40 CaCl₂, 2 KCl and 10 Hepes, pH 7.4 with NaOH. Ca²⁺ currents were recorded in the whole-cell, ruptured-patch configuration. After forming a gigaohm seal in the cell-attached configuration, residual pipette capacitance was compensated using the negative capacitance circuit of the amplifier. The DC resistance of the whole-cell configuration was routinely > 1 G Ω . The steady holding potential was –90 mV. No corrections were made for liquid junction potentials.

Test depolarizations to +30 mV were delivered at 1 Hz unless otherwise indicated. Currents were filtered at 2–10 kHz using the built-in Bessel filter (4-pole low-pass) of an Axopatch 200B amplifier and sampled at 10–50 kHz using a Digidata 1200 analog-to-digital board installed in a Gateway Pentium computer. pCLAMP 7.0 software was used for data acquisition and analysis. Figures were made using Origin (v. 6.0) software.

Linear cell capacitance (C) was determined by integrating the area under the whole-cell capacitance transient, which was evoked by clamping from –90 to –80 mV with the whole-cell capacitance compensation circuit of the amplifier turned off. The mean value of C was 22 ± 0.5 pF (S.E.M.; $n = 164$ cells). Series resistance (R_s) was calculated as $\tau(1/C)$, where τ was the time constant for decay of the whole-cell capacitance transient. Average values of τ and R_s , measured before electronic compensation, were 66 ± 2 μ s and 3.1 ± 0.1 M Ω , respectively ($n = 164$). For these cells, maximal Ca²⁺ current amplitudes were 1193 ± 93 pA (test potential +30 mV). Voltage errors were minimized by using low resistance pipettes and by reducing τ with the series resistance compensation circuit of the amplifier. Typically, series resistance compensation was adjusted to just below the point of oscillation. Following electronic

compensation, the average maximum voltage error was 2.3 ± 0.2 mV. Analysed and illustrated currents were corrected for linear capacitance and leakage currents using $-P/6$ or $-P/4$ subtraction. Ca^{2+} current amplitudes were measured at the time of peak inward current. Statistical comparisons were by one-way ANOVA and Student's unpaired two-tailed or paired t test, as appropriate, with $P < 0.05$ considered significant. Carbachol (CCh) was dissolved directly in the bath solution, and was rapidly applied through a macropipette positioned within ~ 2 mm of the cell being studied. The exchange of solution surrounding the cell was complete within ~ 1 s, based on control experiments in which Ca^{2+} currents were blocked by application of 0.5 mM Cd^{2+} (cf. Melliti *et al.* 1999). Temperature (20 – $23^\circ C$) was continuously monitored using a miniature thermocouple placed in the recording chamber.

RESULTS

Distinct fast and slow components of muscarinic inhibition are reconstituted in HEK293 cells

Figure 1 illustrates whole-cell recordings of N-type Ca^{2+} current from HEK293 cells expressing $\alpha 1B$ Ca^{2+} channel subunits and M1 muscarinic acetylcholine receptors. Initially, experiments were performed using a pipette solution containing 10 mM EGTA. As shown in Fig. 1A, under these recording conditions the expressed N-type current was profoundly inhibited by 1 mM carbachol (CCh). At steady state, $76 \pm 3\%$ ($n = 7$) of the current was inhibited. Recovery from inhibition was slow and incomplete. Inhibition could be prevented by simultaneous

application of 1 mM atropine ($n = 4$; not shown). Furthermore, cells not transfected with receptor plasmid did not respond to CCh ($n = 5$; not shown). Thus, the effects of CCh were mediated through the cloned M1 receptors.

Inhibition had distinct fast and slow components (Fig. 1A). The approximate time constants and amplitudes of these two components were obtained by fitting a biexponential function to plots of Ca^{2+} current amplitude *versus* the time of CCh application. The fast component of inhibition had a time constant of < 1 s. This fast time constant was not well resolved in our experiments because Ca^{2+} currents could not be evoked at a frequency higher than 1 Hz without producing excessive inactivation. However, the slow component was well resolved and had an average time constant of 8 ± 1 s ($n = 7$). The fast and slow components of inhibition reduced the N-type Ca^{2+} current equally, by $38 \pm 6\%$ ($n = 7$) in each case.

High intracellular concentrations of Ca^{2+} buffers have been previously found to suppress slow muscarinic inhibition of N-type Ca^{2+} channels in *rat* SCG neurons (Beech *et al.* 1992; Delmas *et al.* 1998a). In contrast, Ca^{2+} buffers do not suppress slow muscarinic inhibition of N-type current in *mouse* SCG neurons (Shapiro *et al.* 1999). To determine the situation in HEK293 cells, we

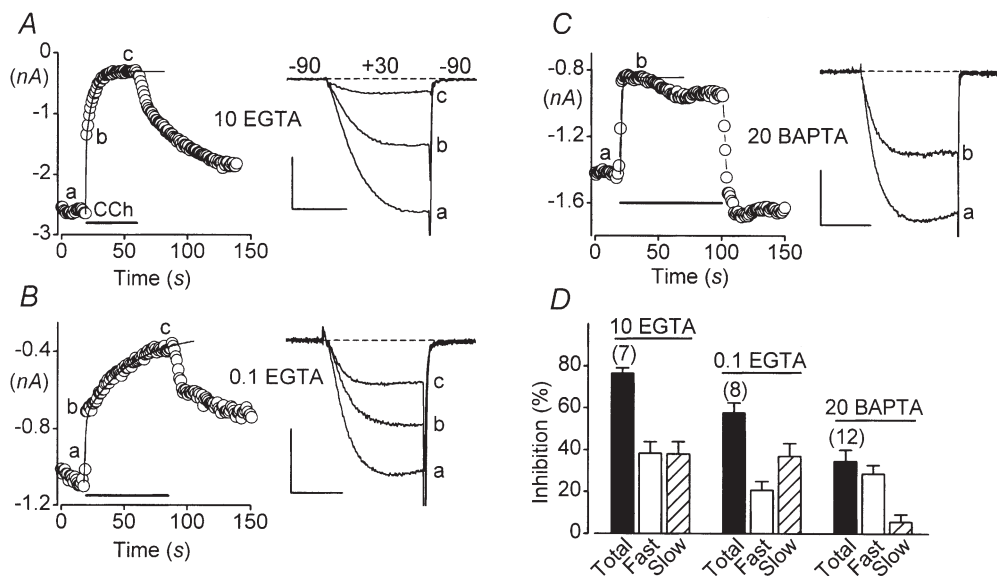


Figure 1. Slow muscarinic inhibition of N-type Ca^{2+} channels is reconstituted in HEK293 cells expressing $\alpha 1B$, $\alpha 2-\delta$ and $\beta 3$ Ca^{2+} channel subunits and M1 receptors

A, rapid application of CCh (indicated by a horizontal bar) reveals distinct fast and slow components of inhibition. The amplitudes of Ca^{2+} currents, evoked at 1 Hz by 10 ms step depolarizations from -90 to $+30$ mV, are shown plotted *vs.* time. A biexponential fit to the time plot is shown as a continuous curve. The pipette solution contained 10 mM EGTA. Linear cell capacitance (C) = 35 pF. Compensated series resistance (R_s) = 1.4 M Ω . Scale: 1 nA, 5 ms. B, the slow component of inhibition is emphasized by low intracellular EGTA. The pipette solution contained 0.1 mM EGTA; conditions otherwise as in A. $C = 22$ pF. $R_s = 2.1$ M Ω . Scale: 0.5 nA, 5 ms. C, the slow component of inhibition is suppressed by high intracellular BAPTA. The pipette solution contained 20 mM BAPTA; conditions otherwise as in A. $C = 23$ pF. $R_s = 1.7$ M Ω . Scale: 0.5 nA, 5 ms. D, summary of results. Error bars represent S.E.M.

switched to a pipette solution containing 0.1 mM EGTA. Total inhibition of the Ca^{2+} current was somewhat reduced (to $58 \pm 5\%$, $n = 8$) under these conditions, apparently because the fast component had a smaller amplitude ($21 \pm 4\%$, $n = 8$) in recordings made with 0.1 mM EGTA ($P = 0.02$; unpaired t test). In contrast, the amplitude of the slow component was unchanged ($37 \pm 6\%$, $n = 8$). However, the time constant of the slow

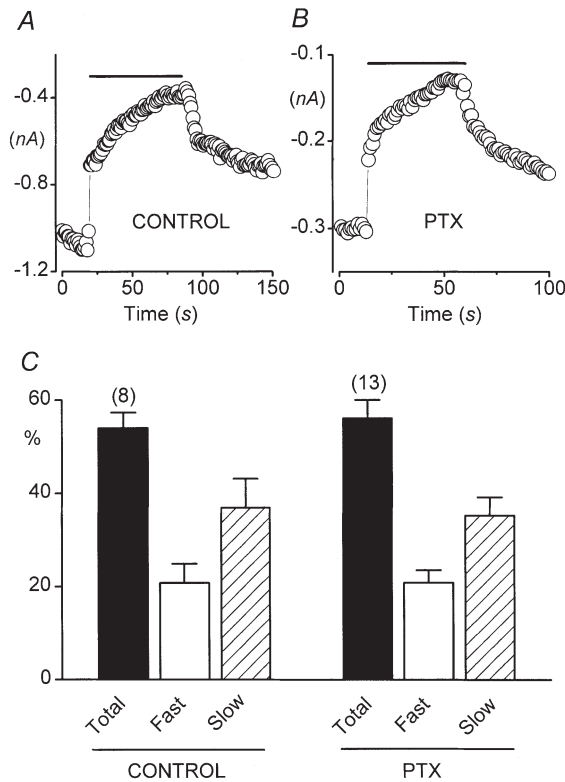


Figure 2. PTX-insensitive G proteins mediate both fast and slow components of inhibition through M1 receptors

Cells were transfected with expression plasmids encoding $\alpha 1\text{B}$, $\alpha 2\text{-}\delta$ and $\beta 3$ Ca^{2+} channel subunits and M1 muscarinic acetylcholine receptors. The pipette solution contained 0.1 mM EGTA. *A*, Ca^{2+} current amplitudes (evoked by 10 ms depolarizations from -90 to $+30$ mV, delivered at 1 Hz) recorded from a control cell (i.e. not treated with PTX) are plotted *vs.* time. Rapid application of CCh (1 mM) is indicated by horizontal bar. $C = 22$ pF. $R_s = 2.1$ M Ω . *B*, a similar plot for a cell incubated with pertussis toxin (PTX; 500 ng ml⁻¹) overnight. $C = 24$ pF. $R_s = 2.3$ M Ω . *C*, relative contributions of fast and slow components of inhibition in PTX-treated and in control (not PTX-treated) cells. In 13 PTX-treated cells, the fast component produced $21 \pm 3\%$ inhibition, and the slow component produced $35 \pm 4\%$ inhibition. In 8 control cells, the fast component produced $21 \pm 4\%$ inhibition, and the slow component produced $37 \pm 6\%$ inhibition. The time constant of slow inhibition was 24 ± 3 s in PTX-treated cells and 21 ± 4 s in control cells.

component was larger (21 ± 4 s, $n = 8$; $P < 0.01$) with low intracellular EGTA. We do not currently know why low EGTA made the fast component smaller and the slow component slower; these effects were not examined further in the present work.

We also performed experiments using a pipette solution containing 20 mM BAPTA, dialysing cells in the whole-cell mode for at least 5 min before CCh was applied. Under these recording conditions, steady-state inhibition of the N-type current was considerably reduced (to $34 \pm 6\%$, $n = 12$). As shown in Fig. 1*C*, high intracellular BAPTA virtually eliminated the slow component of inhibition. Thus, in cells dialysed with BAPTA only $6 \pm 4\%$ ($n = 12$) of the inhibition was attributed to the slow component (Fig. 1*D*). By contrast, the fast component produced $29 \pm 4\%$ inhibition ($n = 12$), not different from its contribution in recordings made with 10 mM EGTA ($P = 0.17$; unpaired t test). These data indicate that high intracellular BAPTA selectively blocked the slow component of reconstituted muscarinic inhibition. This effect of BAPTA agrees with previous results obtained from *rat* SCG neurons (Bernheim *et al.* 1991).

Reconstituted muscarinic inhibition is unaffected by pertussis toxin or staurosporine

M1 receptors preferentially couple to pertussis toxin (PTX)-insensitive G proteins of the $G\alpha_q/11$ subfamily (Felder, 1995). However, previous studies have indicated that M1 receptors can also couple to PTX-sensitive $G\alpha_i/o$ proteins in heterologous expression systems (Stein *et al.* 1988; Offermanns *et al.* 1994). To determine whether $G\alpha_i/o$ contributed to channel inhibition in our experiments, we incubated HEK293 cells with PTX (200–500 ng ml⁻¹) overnight. In these and all subsequent experiments, the 0.1 mM EGTA pipette solution was used. As illustrated in Fig. 2, neither the fast nor the slow component of inhibition was altered by PTX. These results indicate that PTX-insensitive G proteins mediate both fast and slow components of the reconstituted muscarinic inhibition.

Although we did not attempt here to fully elucidate the signalling pathway of slow muscarinic inhibition, we did examine the effects of staurosporine, a broad-spectrum inhibitor of serine/threonine kinases. Cells were exposed to 100 nM staurosporine for 30–60 min before experiments, and staurosporine was present in the bath during Ca^{2+} current recording. In staurosporine-treated cells, the fast component produced $39 \pm 5\%$ ($n = 7$) inhibition, compared with $25 \pm 6\%$ ($n = 8$) in control cells exposed to the vehicle alone (dimethyl sulphoxide, DMSO; $P = 0.12$; unpaired t test). The slow component produced $26 \pm 4\%$ ($n = 7$) inhibition in staurosporine-treated cells, compared with $41 \pm 6\%$ ($n = 8$) inhibition in DMSO-exposed controls ($P = 0.06$; unpaired t test). The time constant of the slow component was similarly unaffected: 17 ± 4 s ($n = 7$) in staurosporine-treated cells and 21 ± 4 s ($n = 8$) in DMSO-treated cells. These results concur with a

previous report that staurosporine does not affect slow muscarinic inhibition of N-type Ca^{2+} channels in SCG neurons (Bernheim *et al.* 1991).

The fast component of inhibition is mediated by $G\beta\gamma$

Previous studies have found that N-type Ca^{2+} channels can be inhibited through two relatively fast pathways. One fast pathway produces a voltage-dependent form of channel inhibition (Bean, 1989; Beech *et al.* 1992; Ikeda & Dunlap, 1999). This pathway is mediated by $G\beta\gamma$ dimers (Herlitze *et al.* 1996; Ikeda, 1996; Dolphin, 1998; Ikeda & Dunlap, 1999). Another fast pathway produces voltage-independent inhibition (Beech *et al.* 1992; Hille, 1994). This second fast pathway appears to be mediated by both $G\beta\gamma$ and $G\alpha_q/11$ subunits (Kammermeier *et al.* 2000).

Fast, voltage-dependent muscarinic inhibition of native N-type Ca^{2+} channels has been attributed to M4 receptors in rat SCG neurons (Bernheim *et al.* 1992; Fernandez-Fernandez *et al.* 1999) and to M2 receptors in mouse SCG neurons (Shapiro *et al.* 1999). Both M2 and M4 receptors signal through PTX-sensitive $G\alpha_i/o$ proteins (Felder, 1995). By contrast, in our cells the fast component of inhibition was clearly PTX insensitive (Fig. 2B). To determine whether this reconstituted fast component was mediated by $G\beta\gamma$, we expressed the carboxyl-terminus fragment (Gly⁴⁹⁵–Leu⁶⁸⁹) of β -adrenergic receptor kinase 1 (denoted β ARK1ct). The β ARK1ct construct sequesters $G\beta\gamma$ dimers (Koch *et al.* 1994), including those released through activation of $G\alpha_q/11$ -coupled M1 receptors (Melliti *et al.* 2000) and M3 receptors (Stehno-Bittel *et al.* 1995).

As shown in Fig. 3B, β ARK1ct completely blocked the fast component of muscarinic inhibition in HEK293 cells. In contrast, the slow component of inhibition was undiminished. Thus, the slow component produced $35 \pm 4\%$ ($n = 13$) inhibition in PTX-treated control cells and $43 \pm 1\%$ ($n = 5$) inhibition in PTX-treated cells expressing β ARK1ct ($P = 0.26$; unpaired t test). These results indicate that the fast component requires signalling by $G\beta\gamma$ dimers, whereas the slow component does not. Since these experiments were conducted using PTX-treated cells, the $G\beta\gamma$ subunits involved in fast inhibition must have originated from PTX-insensitive G proteins (presumably $G\alpha_q/11$).

Reconstituted slow inhibition is voltage independent

The slow muscarinic pathway produces a voltage-independent type of Ca^{2+} channel inhibition in SCG neurons (Beech *et al.* 1992). To determine whether slow muscarinic inhibition is also voltage independent when reconstituted in HEK293 cells, we measured current–voltage (I – V) relationships in the presence and absence of CCh. Cells used in these experiments were cotransfected with β ARK1ct to eliminate the $G\beta\gamma$ -mediated component of inhibition. Under these conditions, only slow inhibition was observed (cf. Fig. 3B and C). As illustrated in Fig. 4A, slow inhibition did not shift the I – V

relationship to either higher or lower test potentials; a shift to higher potentials would be expected for voltage-dependent forms of inhibition. We also looked for evidence of kinetic slowing (Bean, 1989; Luebke & Dunlap, 1994) and prepulse facilitation (Elmslie *et al.* 1990), which are additional distinguishing characteristics of voltage-dependent Ca^{2+} channel inhibition (Ikeda & Dunlap, 1999). Neither kinetic slowing nor prepulse facilitation was detected (Fig. 4B). Instead, N-type currents appeared to inactivate more during steady-state slow inhibition (Fig. 4B). The nature of this extra inactivation (e.g. whether it was ion dependent, voltage dependent, or some combination thereof) was not investigated in the present study. However, enhanced

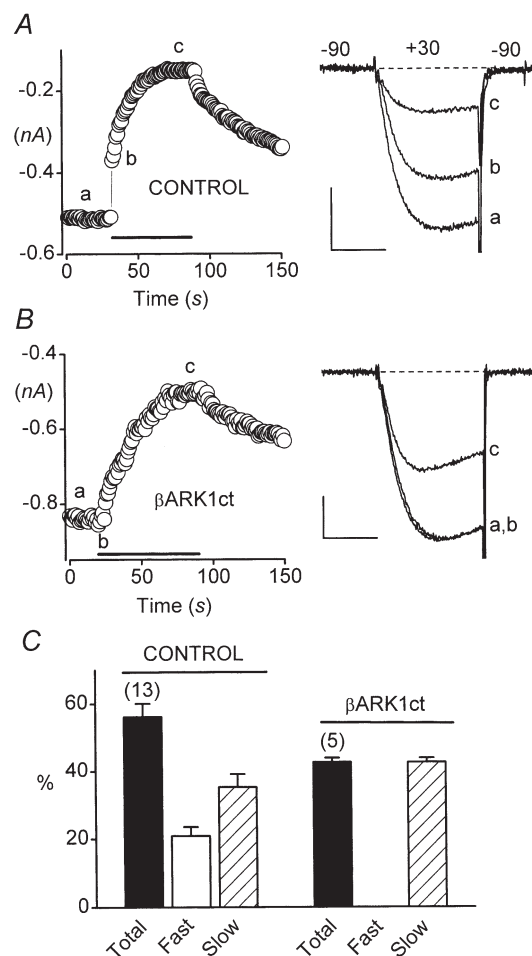


Figure 3. β ARK1ct selectively blocks the fast component of inhibition through M1 receptors

Cells were cotransfected with $\alpha 1B$, $\alpha 2\text{-}\delta$ and $\beta 3$ Ca^{2+} channel subunits and M1 muscarinic acetylcholine receptors, and incubated with PTX (200–500 ng ml⁻¹) overnight before experiments. The pipette solution contained 0.1 mM EGTA. A, distinct fast and slow components of inhibition in a control cell. $C = 34$ pF. $R_s = 2.0$ M Ω . Scale: 0.2 nA, 5 ms. B, the fast component of inhibition is selectively blocked by coexpression of β ARK1ct. $C = 16$ pF. $R_s = 4.1$ M Ω . Scale: 0.2 nA, 5 ms. C, summary of results.

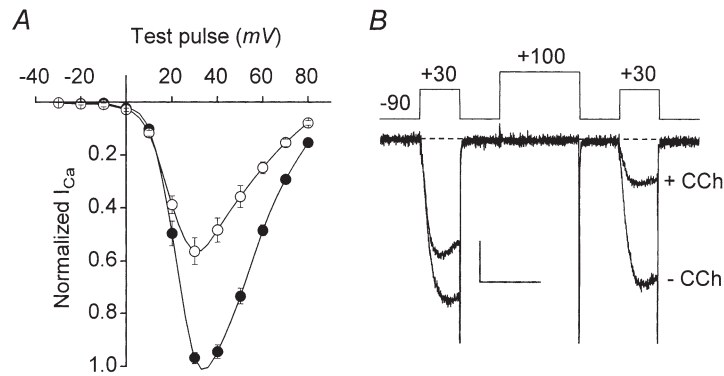


Figure 4. Reconstituted slow muscarinic inhibition is voltage independent

Cells were cotransfected with $\alpha 1B$, $\alpha 2\text{-}\delta$ and $\beta 3$ Ca^{2+} channel subunits, M1 receptors, and βARK1ct , and incubated with PTX ($200\text{--}500\text{ ng ml}^{-1}$) overnight. The pipette solution contained 0.1 mM EGTA. *A*, current–voltage (I – V) relationships of $\alpha 1B$ Ca^{2+} channel currents determined before (\bullet) and during (\circ) steady-state slow inhibition. Control I – V data were obtained (at 0.1 Hz) before CCh application. CCh (1 mM) was then applied, and the onset of slow inhibition was monitored by repetitive depolarizations delivered at 1 Hz . Once slow inhibition had attained steady state, I – V data were again obtained at 0.1 Hz . Symbols represent data from the same eight cells before and after CCh. The average maximal voltage errors were $4.4 \pm 1.4\text{ mV}$ before and $2.6 \pm 0.9\text{ mV}$ after the onset of slow inhibition. *B*, prepulse facilitation is not observed during slow muscarinic inhibition of $\alpha 1B$ Ca^{2+} current. The voltage protocol is illustrated. $C = 18\text{ pF}$, $R_s = 1.8\text{ M}\Omega$. Scale: 0.2 nA , 15 ms .

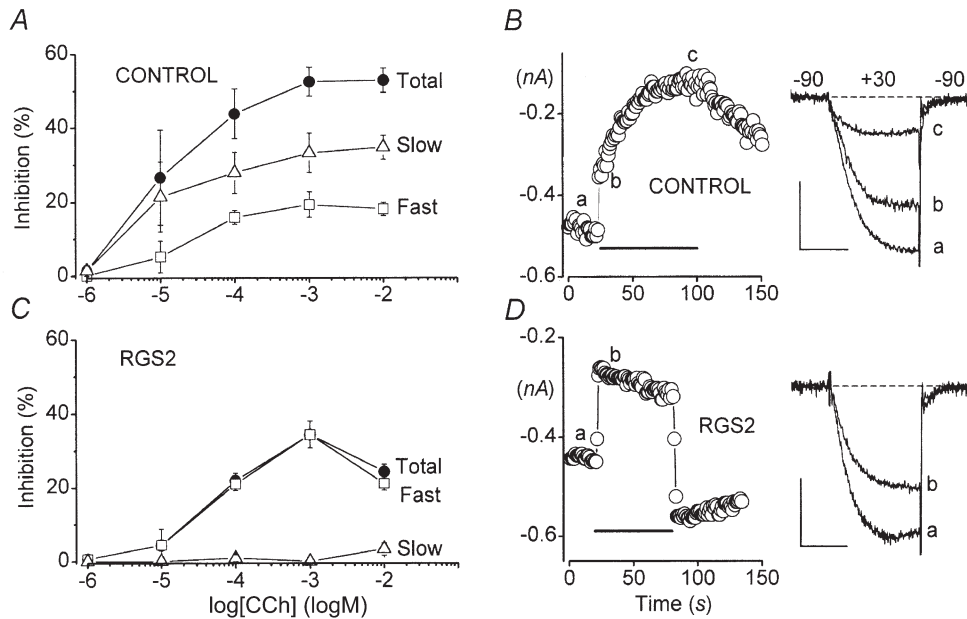


Figure 5. RGS2 selectively blocks the slow component of inhibition through M1 receptors

Control cells were cotransfected with $\alpha 1B$, $\alpha 2\text{-}\delta$ and $\beta 3$ Ca^{2+} channel subunits and M1 receptors. RGS2 cells were additionally transfected with EGFP–RGS2. All cells were incubated with PTX ($200\text{--}500\text{ ng ml}^{-1}$) overnight. The pipette solution contained 0.1 mM EGTA. *A*, inhibition of $\alpha 1B$ Ca^{2+} current in control cells in response to various concentrations of CCh. Dose–response data for the slow component of inhibition (Δ), the fast component of inhibition (\square) and total inhibition (\bullet) are plotted separately. Symbols represent the means \pm S.E.M. of 4–8 cells. *B*, representative experiment in a control cell. $C = 34\text{ pF}$, $R_s = 1.8\text{ M}\Omega$. Scale: 0.2 nA , 5 ms . *C*, dose–response data for RGS2-expressing cells. Symbols represent the means \pm S.E.M. of 3–11 cells. *D*, representative experiment in an RGS2-expressing cell. $C = 18\text{ pF}$, $R_s = 1.5\text{ M}\Omega$. Scale: 0.2 nA , 5 ms .

inactivation may be an important, previously unappreciated effect of the slow muscarinic pathway. Altogether, the slow muscarinic pathway failed to produce any of the classic signs of voltage-dependent Ca^{2+} channel inhibition. These results indicate that slow muscarinic inhibition of N-type channels is voltage independent in HEK293 cells, as it is in neurons.

RGS2 selectively blocks the slow component of muscarinic inhibition

Slow muscarinic inhibition of N-type Ca^{2+} channels involves a $G\alpha_q/11$ -dependent signalling pathway in rodent SCG neurons (Delmas *et al.* 1998a; Haley *et al.* 1998, 2000). RGS2 is a regulator of G protein-signalling protein that preferentially interacts with $G\alpha_q/11$ (Heximer *et al.* 1997, 1999; Ingi *et al.* 1998). The expression of neuronal RGS2 can be upregulated by various stimuli, including activation of muscarinic receptors (Song *et al.* 1999). Importantly, Oliveira-dos-Santos *et al.* (2000) have shown that RGS2 is a critical regulator of neuronal function *in vivo*. Altogether, these considerations make RGS2 a logical candidate as a potential regulator of the slow muscarinic pathway. We therefore asked whether RGS2 could influence slow muscarinic inhibition in HEK293 cells. As a first step in these experiments, we determined the complete dose-response relationship for CCh-induced inhibition of expressed N-type Ca^{2+} current. As anticipated, the extent of inhibition increased at higher agonist concentrations, reaching saturation at ~ 1 mM CCh (Fig. 5A). The dose-response curves for fast and slow components of inhibition were plotted separately, using data derived from the biexponential fits. Fast and slow inhibition displayed similar dose-response profiles in control cells (Fig. 5A).

Coexpression of RGS2 completely blocked the slow component of inhibition (Fig. 5C). The remaining inhibition in RGS2-expressing cells thus appeared entirely fast (Fig. 5D). In response to 1 mM CCh, the residual fast component produced $34 \pm 4\%$ ($n = 8$) inhibition in RGS2-expressing cells, significantly greater than the $19 \pm 3\%$ ($n = 8$) inhibition attributed to the fast component in control cells ($P = 0.01$; unpaired *t* test). This comparison suggests that the fast component of inhibition was actually increased in RGS2-expressing cells. RGS2 also appeared to accelerate recovery of N-type current from inhibition following CCh washout (Fig. 5D). However, it is important to note that this kinetic effect is unlikely to have resulted from RGS2 acting as a GAP because accelerated GTP hydrolysis should have speeded formation of heteromeric $G\alpha\beta\gamma$ and should have reduced the fast $G\beta\gamma$ -mediated component of inhibition as well as the slow $G\alpha$ -mediated component. As described above, the fast $G\beta\gamma$ -mediated component of inhibition appeared to be increased, rather than decreased, by RGS2.

A modest but significant kinetic slowing of the CCh-inhibited current was noted in RGS2-expressing cells

(Fig. 5D). Thus, at a test potential of +30 mV the average time constant for activation of N-type current in seven RGS2-expressing cells was 1.8 ± 0.1 ms before application of CCh and 2.1 ± 0.1 ms during steady-state inhibition ($P = 0.02$; paired *t* test). In contrast, no kinetic slowing was detected in currents recorded from eight control cells (time constants = 2.2 ± 0.2 ms before and 2.3 ± 0.2 ms after CCh application). The appearance of kinetic slowing

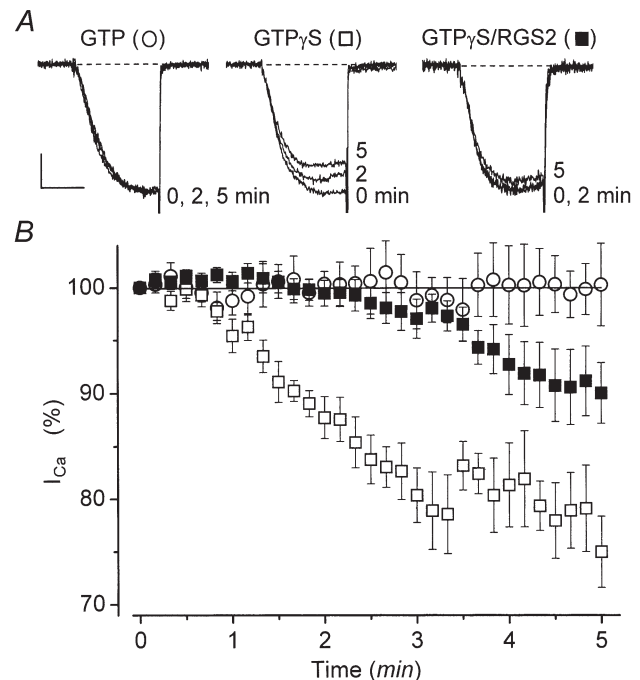


Figure 6. RGS2 delays and attenuates GTP γ S-mediated inhibition

Cells were transfected with $\alpha 1B$, $\alpha 2\text{-}\delta$ and $\beta 3$ Ca^{2+} channel subunits, M1 receptors, and $\beta ARK1ct$, with or without RGS2. All cells were incubated with PTX ($200\text{--}500$ ng ml $^{-1}$) overnight before experiments. The pipette solution contained 0.1 mM EGTA and either 0.3 mM GTP (○) or 0.3 mM GTP γ S (■ or □).

A, representative Ca^{2+} currents recorded at selected times. Currents were evoked at 0.1 Hz by 10 ms step depolarizations from -90 to $+30$ mV. The scale bar corresponds to 5 ms and 0.3 nA (GTP), 0.4 nA (GTP γ S) or 0.2 nA (GTP γ S/RGS2). GTP cell: $C = 22$ pF, $R_s = 2.4$ M Ω ; GTP γ S: $C = 19$ pF, $R_s = 2.6$ M Ω ; GTP γ S/RGS2: $C = 18$ pF, $R_s = 1.7$ M Ω . B, normalized Ca^{2+} current amplitudes plotted as a function of time. Ca^{2+} current amplitudes typically increased (i.e. 'run up') during the initial 2–3 min following break-in. Time zero (0) was taken as the earliest time at which run-up was complete and the Ca^{2+} current amplitude had stabilized. Symbols represent means \pm S.E.M. for 8 (GTP), 8 (GTP γ S) and 14 (GTP γ S/RGS2) cells up to 3 min. After 3 min, symbols represent means \pm S.E.M. for 4 (GTP), 3 (GTP γ S) and 9 (GTP γ S/RGS2) cells. Initial Ca^{2+} current densities were 57 ± 23 pA pF $^{-1}$ ($n = 8$ cells; GTP), 65 ± 23 pA pF $^{-1}$ ($n = 8$ cells; GTP γ S), and 71 ± 20 pA pF $^{-1}$ ($n = 14$ cells; GTP γ S/RGS2).

in RGS2-expressing cells may be significant if it reflects increased concentrations of free $G\beta\gamma$ subunits and/or decreased signalling by $G\alpha$ (see Discussion).

RGS2 delays and attenuates GTP γ S-mediated inhibition

Previous studies have demonstrated that RGS2 can function, in solution assays, as a GAP for a GTPase-deficient mutant (R183C) of $G\alpha_q$ (Ingi *et al.* 1998). RGS2 has also been shown to interfere with signalling by wild-type $G\alpha_q/11$ that has been activated by GTP γ S (Heximer *et al.* 1997, 1999). Because GTP γ S cannot be hydrolysed by $G\alpha$ subunits, this latter effect of RGS2 cannot be attributed to GAP activity. Instead, RGS2 is thought to function as an effector antagonist by binding to GTP γ S- $G\alpha$ and physically blocking its interactions with downstream effector molecules.

The selective block of slow inhibition (Fig. 5) suggests that RGS2 behaved primarily as an effector antagonist in our experiments. To further assess this possibility, we used GTP γ S to trigger inhibition of the N-type Ca^{2+} current. For these experiments, cells were cotransfected with β ARK1ct to prevent $G\beta\gamma$ -mediated inhibition and then incubated with PTX overnight (200–500 ng ml⁻¹), under the assumption that PTX would prevent GTP γ S-induced signalling by $G\alpha_i/o$, as indicated by previous studies (Ito *et al.* 1991; Delmas *et al.* 1998b). As shown in Fig. 6, dialysis with GTP γ S produced a significant, slowly developing inhibition of the N-type Ca^{2+} current. This GTP γ S-mediated slow inhibition closely resembled that observed in PTX-treated rat SCG neurons (Delmas *et al.* 1998b). No inhibition was observed in control cells dialysed with GTP (Fig. 6). Importantly, in RGS2-expressing cells the onset of inhibition was clearly delayed, and its final magnitude was significantly attenuated (Fig. 6). These results strongly support the view that RGS2 functioned primarily as an effector antagonist, rather than as a GAP in our experiments.

DISCUSSION

Slow muscarinic inhibition of N-type Ca^{2+} channels is reconstituted in HEK293 cells

Here we show for the first time that slow muscarinic inhibition of N-type Ca^{2+} channels can be reconstituted in a mammalian cell line. The time course of this reconstituted inhibition (Fig. 1) is comparable to the time course of slow muscarinic inhibition in SCG neurons (Bernheim *et al.* 1991; Hille, 1994). Additionally, slow inhibition is blocked by high intracellular BAPTA in HEK293 cells as in rat SCG neurons (Beech *et al.* 1992; but see Shapiro *et al.* 1999). We also find that the slow muscarinic pathway produces a voltage-independent form of Ca^{2+} channel inhibition in HEK293 cells (Fig. 4), consistent with previous results from neurons (Beech *et al.* 1992). Finally, the reconstituted inhibition is insensitive to staurosporine, in agreement with previous

observations in native cells (Bernheim *et al.* 1991). Thus, slow muscarinic inhibition of N-type Ca^{2+} channels reconstituted in HEK293 cells appears to closely resemble slow muscarinic inhibition of native N-type channels in rat SCG neurons.

Recently, Shapiro *et al.* (2000) reconstituted slow muscarinic inhibition of M-type K^+ channels in tsA201 cells. This reconstituted channel modulation was also insensitive to staurosporine (Shapiro *et al.* 2000). Slow muscarinic inhibition of N-type Ca^{2+} channels and M-type K^+ channels is thought to proceed through similar pathways (Hille, 1994). However, recent data from $G\alpha_q$ -deficient mice indicate that different $G\alpha$ subunits mediate slow muscarinic inhibition of these two channel types (Haley *et al.* 2000). By demonstrating that slow muscarinic inhibition of N-type Ca^{2+} channels can be reconstituted in HEK293 cells, our present work (together with that of Shapiro *et al.* 2000) should contribute to further delineation of slow muscarinic signalling pathways.

The fast component of inhibition is mediated by $G\beta\gamma$ dimers presumably released from $G\alpha_q/11$

Most previous studies have concluded that fast inhibition of N-type Ca^{2+} channels is mediated by PTX-sensitive G proteins (Beech *et al.* 1992; Bernheim *et al.* 1992; Fernandez-Fernandez *et al.* 1999; Ikeda & Dunlap, 1999; Shapiro *et al.* 1999). In contrast, PTX did not diminish the fast component of inhibition in our cells (Fig. 2). Additionally, we found that the fast component was completely blocked by β ARK1ct (Fig. 3), whereas it was undiminished by RGS2 (Fig. 5). M1 receptors are known to preferentially couple to $G\alpha_q/11$ (Felder, 1995). Taken altogether, these results suggest that fast inhibition was mediated by $G\beta\gamma$ dimers released from $G\alpha_q/11$ in our cells. A fast component of muscarinic inhibition was previously observed in SCG neurons treated with N-ethylmaleimide to inactivate PTX-sensitive G proteins (Zhou *et al.* 1997). Thus, $G\beta\gamma$ from $G\alpha_q/11$ can probably also mediate fast inhibition of native N-type Ca^{2+} channels in neurons (see also Kammermeier *et al.* 2000).

The fast component of inhibition was increased, rather than decreased, in RGS2-expressing cells (compare Fig. 5A and C). While the mechanism underlying this increase is not known, previous results suggest two possible explanations. One is that $G\alpha$ somehow blocks the actions of $G\beta\gamma$ on N-type Ca^{2+} channels (Kammermeier & Ikeda, 1999; Kammermeier *et al.* 2000), such that effects of $G\beta\gamma$ (e.g. fast inhibition) are more strongly manifested following sequestration of $G\alpha$ by RGS2. A second (not mutually exclusive) possibility is that more $G\beta\gamma$ dimers are available to mediate fast inhibition in RGS2-expressing cells. This second possibility was first advanced by Bünemann & Hosey (1998) to explain observations in cells overexpressing RGS4. Recent experiments by Mark *et al.* (2000) support this second possibility. Both explanations are compatible with the

idea that RGS2 behaved as an effector antagonist rather than as a GAP, because prolonged binding of RGS2 to G α (without accelerating GTP hydrolysis) should block signalling by G α without interfering with signalling by its G $\beta\gamma$ subunit, and should also increase the availability of G $\beta\gamma$.

RGS2 functioned as an effector antagonist to selectively block slow muscarinic inhibition

RGS2 belongs to a large protein family, certain members of which can act as GAPs for some G α subunits (Berman & Gilman, 1998; De Vries *et al.* 2000). Previous studies have demonstrated that RGS2 preferentially interacts with G $\alpha_q/11$. Thus, in solution assays RGS2 binds to G $\alpha_q/11$ but not to G α_i , G α_o , G α_s or G $\alpha_{12/13}$ (Heximer *et al.* 1997). Also in solution assays, RGS2 can act as a GAP for a GTPase-deficient mutant of G α_q (Ingi *et al.* 1998). In phospholipid vesicles, RGS2 can function as an effector antagonist to prevent G $\alpha_q/11$ from stimulating phospholipase C in the presence of GTP γ S or aluminium fluoride (Heximer *et al.* 1997, 1999; Yan *et al.* 1997). RGS2 blocks G α_q -mediated signalling in reconstituted phospholipid vesicles more effectively than RGS4 (Heximer *et al.* 1997, 1999), consistent with the notion that G $\alpha_q/11$ preferentially interacts with RGS2. In intact cells, RGS2 reduced activation of MAP kinases by wild-type G $\alpha_q/11$ (Ingi *et al.* 1998). However, for these latter experiments it is unknown whether RGS2 functioned as a GAP or as an effector antagonist, because signalling by G $\alpha_q/11$ and G $\beta\gamma$ could not be distinguished as they were in our present study.

We found that RGS2 blocked the slow component of muscarinic inhibition without reducing the fast component (Fig. 5). Our experiments show that the fast component of inhibition is attributable to G $\beta\gamma$ (Fig. 3*B* and *C*), whereas the slow component is mediated by G α (Fig. 5). Our results therefore indicate that RGS2 blocked signalling by G α (presumably G $\alpha_q/11$) without reducing signalling by its G $\beta\gamma$ dimer. This selective block of G α is unlikely to be explained by GAP activity, because simply accelerating GTP hydrolysis should have reduced signalling by both G α and G $\beta\gamma$ and should have attenuated both slow and fast components of inhibition. However, the selective block of slow inhibition is consistent with RGS2 functioning as an effector antagonist. This interpretation is supported by our finding that RGS2 delayed and attenuated GTP γ S-mediated slow inhibition (Fig. 6). That RGS2 did not completely prevent the effects of GTP γ S (Fig. 6) might be expected if the slow pathway involves considerable amplification, and if only a few activated G α subunits need escape interaction with RGS2 to produce channel inhibition. Furthermore, GTP γ S-mediated slow inhibition may be partially attributable to RGS2-insensitive G α subunits.

The interpretation that RGS2 functioned primarily as an effector antagonist is also supported by the presence of kinetic slowing in RGS2-expressing cells (Fig. 5*D*).

Kinetic slowing is a hallmark of G $\beta\gamma$ -mediated, voltage-dependent Ca²⁺ channel inhibition (Ikeda & Dunlap, 1999). The appearance of kinetic slowing in RGS2-expressing cells (Fig. 5*D*) may reflect an increased pool of free G $\beta\gamma$ dimers, resulting from RGS2 acting as an effector antagonist rather than as a GAP (see above). Alternatively, kinetic slowing may have been uncovered in RGS2-expressing cells following sequestration of G α , if G α can block the effects of G $\beta\gamma$ as recently proposed by Kammermeier *et al.* (2000).

Because the signalling pathway of slow muscarinic inhibition has not been fully elucidated, the identity of the effector antagonized by RGS2 remains unknown. However, it is probably not the N-type channel itself. More likely, the effector is an enzyme that triggers production of a diffusible messenger (also unidentified) that somehow causes slow muscarinic inhibition.

Previous studies have shown that RGS proteins can shift the dose–response curve for inhibition of N-type Ca²⁺ channels to higher agonist concentrations (Melliti *et al.* 1999; Jeong & Ikeda, 2000). RGS proteins can also accelerate recovery of N-type channels from inhibition (Jeong & Ikeda, 1998, 2000; Melliti *et al.* 1999). In these previous studies, Ca²⁺ channels were inhibited through PTX-sensitive G proteins, and the examined RGS proteins appeared to act principally as GAPs. By contrast, in the present study Ca²⁺ channels were inhibited through PTX-insensitive G proteins (presumably G $\alpha_q/11$) and RGS2 appeared to function primarily as an effector antagonist. Recently, RGS2 was shown to function as an effector antagonist in blocking muscarinic stimulation, but not inhibition, of R-type Ca²⁺ channels (Melliti *et al.* 2000). In this study, channel modulation was generated through PTX-insensitive G proteins (presumably G $\alpha_q/11$) coupled to M1 receptors. Taken altogether, these observations suggest that RGS proteins behave either as GAPs or as effector antagonists depending upon the type of G α subunit involved.

In summary, we have demonstrated that slow muscarinic inhibition of N-type channels can be reconstituted in HEK293 cells. Furthermore, we have shown that RGS2 can selectively block slow muscarinic inhibition of N-type Ca²⁺ channels, without reducing fast inhibition generated through the same receptor. Our results suggest that RGS2 may serve as a physiological regulator of the slow muscarinic pathway. RGS2 may not be unique in this regard, as other RGS proteins (e.g. RGS4; Heximer *et al.* 1997) and phospholipase C- β 1 (Berstein *et al.* 1992) can also bind G $\alpha_q/11$. Significantly, the effects of RGS2 in our experiments seem unlikely to be explained by GAP activity. Instead, our results suggest that RGS2 functioned primarily as an effector antagonist. This conclusion may have general significance for understanding the physiological roles of RGS proteins. Because RGS2 preferentially interacts with G $\alpha_q/11$, it could potentially modify signalling through any G $\alpha_q/11$ -mediated

signalling pathway. Recently, Jeong & Ikeda (2000) demonstrated that endogenous RGS proteins influence inhibition of N-type channels in SCG neurons. Although it remains unknown which RGS proteins are constitutively expressed in these native cells, our present data suggest that RGS2 is not, because slow muscarinic inhibition is readily observable in SCG neurons. However, it is important to note that expression of neuronal RGS2 can be upregulated by seizure-like electrical activity (Ingi *et al.* 1998), pharmacological agents such as amphetamines (Burchett *et al.* 1998), or activation of muscarinic receptors (Song *et al.* 1999). We speculate that blockage of the slow muscarinic pathway, either through constitutive expression of RGS2 or its transient upregulation in response to specific stimuli, could profoundly alter neuronal function.

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